Acta Pharmaceutica Sinica B 2015;5(5):461–466

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Acta Pharmaceutica Sinica B

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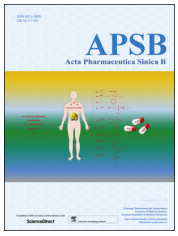

ORIGINAL ARTICLE

Determination of ginsenoside compound K in human plasma by liquid chromatography–tandem mass spectrometry of lithium adducts

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Received 19 March 2015; received in revised form 19 April 2015; accepted 7 May 2015

KEY WORDS

LC – MS/MS;
Ginsenoside compound K;
Lithium adduct ion;
Pharmacokinetics

Abstract Ginsenoside compound K (GCK), the main metabolite of protopanaxadiol constituents of *Panax ginseng*, easily produces alkali metal adduct ions during mass spectrometry particularly with lithium. Accordingly, we have developed a rapid and sensitive liquid chromatography–tandem mass spectrometric method for analysis of GCK in human plasma based on formation of a lithium adduct. The analyte and paclitaxel (internal standard) were extracted from 50 μL human plasma using methyl *tert*-butyl ether. Chromatographic separation was performed on a Phenomenex Gemini C18 column (50 mm × 2.0 mm; 5 μm) using stepwise gradient elution with acetonitrile–water and 0.2 mmol/L lithium carbonate at a flow rate of 0.5 mL/min. Detection was performed in the positive ion mode using multiple reaction monitoring of the transitions at *m/z* 629→449 for the GCK-lithium adduct and *m/z* 860→292 for the adduct of paclitaxel. The assay was linear in the concentration range 1.00–1000 ng/mL ($r^2 > 0.9988$) with intra- and inter-day precision of ±8.4% and accuracy in the range of −4.8% to 6.5%. Recovery, stability and matrix effects were all satisfactory. The method was successfully applied to a pharmacokinetic study involving administration of a single GCK 50 mg tablet to healthy Chinese volunteers.

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Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

http://dx.doi.org/10.1016/j.apsb.2015.06.003

1. Introduction

Ginseng is a traditional Chinese medicine widely used in Asian countries. Ginsenoside compound K (GCK; 20-*O*-*β*-(D-glucopyranosyl)-20(*S*)-protopanaxadiol; Fig. 1) is a metabolite of protopanaxadiol ginsenosides, such as Rb_1, Rb_2, and Rc[1–3] produced by human intestinal bacteria after oral ingestion. Previous studies have shown that GCK exerts stronger anti-colorectal cancer activity than Rb_1[4] and exhibits antiapoptotic[5], antidiabetic[6], antiallergic[7], anti-inflammatory[8], and antiaging activities[9]. Despite these promising beneficial effects, drug development of GCK is compromised by the lack of a rapid and reliable method for its quantitation in *in vivo* and *in vitro* studies.

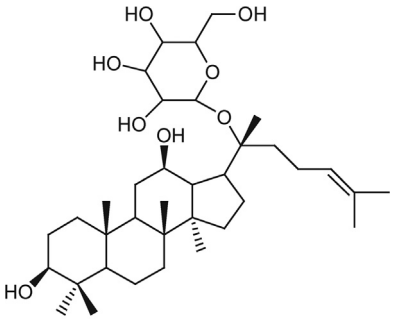
Several bioanalytical methods have been developed for GCK, but they were usually aimed at simultaneous determination of ginsenosides which are both time-consuming and relatively insensitive. The methods include high-performance liquid chromatography with ultraviolet detection (HPLC–UV)[10], ultra-performance liquid chromatography coupled to time-of-flight mass spectrometry (UPLC/TOF–MS)[11], and liquid chromatography–tandem mass spectrometry (LC–MS/MS)[12]. One LC–MS/MS method[13] using the negative ion mode has been reported for determination of GCK in human plasma, but in our hands, the response of the $[M-H]^-$ ion was negligible.

As a neutral compound, GCK is not easily ionized during MS and gives only a weak response in both positive and negative ion modes. A possible method to improve the MS response in the positive ion mode is to add alkali metal salts or alkyl ammonium salts[14–17]. This was the case for the determination of decitabine in human plasma by hydrophilic interaction liquid chromatography–tandem mass spectrometry (HILIC–MS/MS)[18] and for paclitaxel and hydroxylated metabolites in rat plasma by LC–MS/MS[19], both assays using lithium adduct detection. In the present study, we have used this technology to determine GCK in human plasma using paclitaxel as internal standard (IS) and applied the method to a pharmacokinetic study of GCK in healthy Chinese volunteers.

2. Experimental

2.1. Chemicals and additives

Materials and their suppliers were as follows: GCK (chemical purity 98.9%), Zhejiang Hisun Pharmaceutical Co., Ltd. (Zhejiang, China); paclitaxel (chemical purity 98.0%), Jiangsu Hengrui Medicine Co., Ltd. (Jiangsu, China); methyl tertiary butyl ether (chemical purity 99.0%), Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); lithium carbonate (chemical purity 99%), Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); HPLC-grade methanol and acetonitrile, Sigma–Aldrich (St. Louis, USA). Human blank plasma was provided by Shuguang Hospital (Shanghai, China) and deionized water purified using a Millipore Milli-Q Gradient Water Purification System (Molsheim, France).



$C_{36}H_{62}O_8$ (622.87)

Figure 1 Structure of GCK.

2.2. Instrumentation

The HPLC system consisting of two LC-30AD pumps and an SIL-30AC autosampler (Shimadzu, Kyoto, Japan) was coupled to an AB Sciex Qtrap 5500 System (Applied Biosystems, Foster City, USA) equipped with a TurboIonSpray ionization interface. Analyst Version 1.5.2 (Applied Biosystems) was used for data acquisition. Centrifugation employed a CT 15 RE high-speed desktop centrifuge (Hitachi, Tokyo, Japan).

2.3. LC–MS/MS conditions

Lithium adducts of GCK and IS were separated from matrix components on a Phenomenex Gemini C18 column (50 mm × 2.0 mm, 5 μm) using stepwise gradient elution with 0.2 mmol/L lithium carbonate in water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.5 mL/min. The gradient program was 0–0.5 min 50% B; 0.5–1.8 min 80% B; 1.8 min 50% B. The autosampler was maintained at 4 °C and the injection volume was 5 μL.

The electrospray ionization (ESI) source of the mass spectrometer was operated in the positive ion mode. Detection was by multiple reaction monitoring (MRM) of the transitions at *m/z* 629→449 for the lithium adduct ion of GCK and *m/z* 860→292 for that of paclitaxel with a dwell time of 100 ms. Optimal MS parameters were as follows: curtain gas 35 psi; collision gas medium; nebulizer gas (GS_1) and turbo gas (GS_2) 50 psi; ion spray voltage 5000 V; source temperature 400 °C; collision energies 44 and 35 eV for GCK and IS, respectively; declustering potential 40 V for both GCK and IS.

2.4. Preparation of calibration standards and quality control (QC) samples

Stock solutions of GCK (1 mg/mL) were prepared in methanol and stored at 4 °C. Standard solutions were prepared by serial dilution with 50% aqueous methanol to concentrations of 50.0, 150, 500, 1500, 5000, 15,000 and 50,000 ng/mL. Calibration standards for GCK were prepared by spiking 980 μL blank human plasma with 20 μL of the respective standard solutions to give final concentrations of 1.00, 3.00, 10.0, 30.0, 100, 300 and 1000 ng/mL. All solutions were stored at −80 °C until required. LLOQ (lower limit of quantitation), low, medium and high QC samples were prepared in a similar way at concentrations of 1.00, 2.00, 30.0, and 800 ng/mL respectively and also stored at −80 °C. An IS stock solution in methanol was diluted with 50% aqueous methanol to obtain a 200 ng/mL working IS solution before storage at 4 °C.

2.5. Sample preparation

200 μL water and 50 μL IS working solution were added to an aliquot of plasma. After vortex-mixing, the sample was extracted by shaking with 500 μL methyl *tert*-butyl ether for 5 min and subsequently centrifuged at 14,000 × *g* for 5 min at room temperature (20 °C). The organic phase was transferred to another

tube and evaporated to dryness at 40 °C under a stream of nitrogen in a TurboVap evaporator (Zymark, Hopkinton, USA). The residue was reconstituted in 200 µL 50% aqueous methanol and injected into the LC−MS/MS system.

2.6. Assay validation

This was carried out in accordance with EMA guidelines[20].

2.6.1. Selectivity

Selectivity was evaluated by analyzing three replicates of six batches of human non-hemolyzed plasma and one batch of human hemolyzed plasma (prepared by adding 2% lysed whole blood to plasma) at the LLOQ level. The assay was considered free of interference at the retention time of GCK and IS if the response was <20% of that of the LLOQ for GCK and <5% of that of the IS.

2.6.2. Linearity

This was evaluated by least squares analysis of calibration curves based on peak area ratios of analyte to IS (y) weighted using $1/x^2$ (x, concentration). Linearity was accepted when the correlation coefficient (r^2) was >0.99 and back-calculated concentrations were ±15% of nominal values (±20% at the LLOQ).

2.6.3. Precision and accuracy

Intra- and inter-day precision and accuracy were determined by analyzing six replicate QC samples on three successive days. Concentrations were determined using duplicate calibration curves prepared independently. Precision as relative standard deviation (RSD) and accuracy as relative error (RE) were considered acceptable if values were <15% and ±15% (±20% at the LLOQ), respectively.

2.6.4. Recovery and matrix effects

Recovery of analyte and IS was determined by comparing peak areas of extracted low, medium and high QC samples with those of extracted blank plasma spiked with solutions at corresponding concentrations. Matrix effects were evaluated by comparing peak areas of spiked extracts of six samples of blank non-hemolyzed plasma and one sample of hemolyzed plasma with those of three replicates of water spiked with GCK at corresponding concentrations and IS. Precision for assay of the seven samples should be <15%.

2.6.5. Stability

Stability of GCK in human plasma was evaluated by assay in triplicate of plasma samples at low and high concentrations (2.00 and 800 ng/mL) stored under the following conditions: −80 °C for 32 days; after three freeze–thaw cycles from −80 °C; room temperature for 6 h. The stability of processed samples in the autosampler at 4 °C for 24 h was also evaluated. Finally, stability of the GCK stock solution at room temperature for 6 h and that of the GCK stock solution and IS working solution in a refrigerator at 4±3 °C for 32 days were tested. Stability in plasma and solutions was considered acceptable if concentrations were <15% and ≤10%, respectively, of initial levels.

2.6.6. Carry-over

Carry-over was assessed by assay of a double blank sample (no GCK, no IS) immediately after assay of the highest calibration standard (1000 ng/mL). It was considered acceptable for GCK if the response was <20% of the LLOQ standard and for IS if <5% of the control zero sample.

2.7. Pharmacokinetic study

The method was applied to a pharmacokinetic study involving oral administration of a GCK 50 mg tablet (Hisun Pharmaceutical Co., Ltd., Zhejiang, China) with 200 mL water to 12 healthy Chinese volunteers (six males, six females, aged 25–32 years). The study was approved by the Ethics Committee of the Third Xiangya Hospital, Central South University (Changsha, China). Blood samples (4 mL) were collected into sodium heparin-containing tubes predose and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10, 12 and 24 h postdose. Plasma was collected after centrifugation at 2000 × g for 10 min and stored at −80 °C until analysis.

3. Results and discussion

3.1. Mass spectrometric conditions

In the early stages of method development, ionization of GCK was investigated in both negative and positive ion modes. Because GCK is a neutral compound with low polarity, its MS response in ESI was weak under both conditions. However, in the negative ion mode, the responses of the solvent adduct ions $[M+HCOO]^-$ (m/z 667) and $[M+CH_3COO]^-$ (m/z 681), produced in the presence of formic acid and acetic acid, respectively, were much stronger than that of $[M-H]^-$ (m/z 621). Unfortunately the $[M+HCOO]^-$ ion was unstable and gave highly variable response between two calibration curve determinations.

In the positive ion mode, $[M+H]^+$ was not detected and the most abundant ions produced from GCK were $[M+Na]^+$ followed by $[M–Glu–2H_2O+H]^+$. The response of the $[M–Glu–2H_2O+H]^+$ ion at 1 ng/mL GCK was too weak for quantitation, and after adjusting either the temperature or ejection voltage source, the fragment ions of $[M+Na]^+$ were unstable. In the presence of lithium ions, both lithium and sodium adducts were observed but only the $[M+Li]^+$ ion fragmented easily to produce stable product ions with substantial intensity. Therefore, the positive ion mode was selected to quantitate GCK and IS using their most stable fragment ions at m/z 449 and 292 respectively in the MRM mode. The product ion spectra of GCK and IS are presented in Fig. 2.

3.2. Chromatographic conditions

Several C18 HPLC columns were evaluated including the ASB and Agilent XDB columns but, in all cases, the IS gave poor retention even at low acetonitrile or methanol levels in the mobile phase. However, the Phenomenex Gemini C18 column (50 mm × 2.0 mm, 5 µm) gave adequate retention, selectivity and peak shape.

The MS responses of analyte (m/z 425) and IS remained intense when methanol or acetonitrile was used as the organic modifier. However, acetonitrile gave shorter retention time. Initially 5 mmol/L ammonium acetate was evaluated as solvent A, but the response of the analyte was three-fold lower than using water. Similarly, the inclusion of either formic acid or acetic acid produced a two-fold reduction in analyte response. The previously reported assay of 20(*S*)-protopanaxadiol in rat plasma[17] employed 25 mmol/L lithium acetate, but for

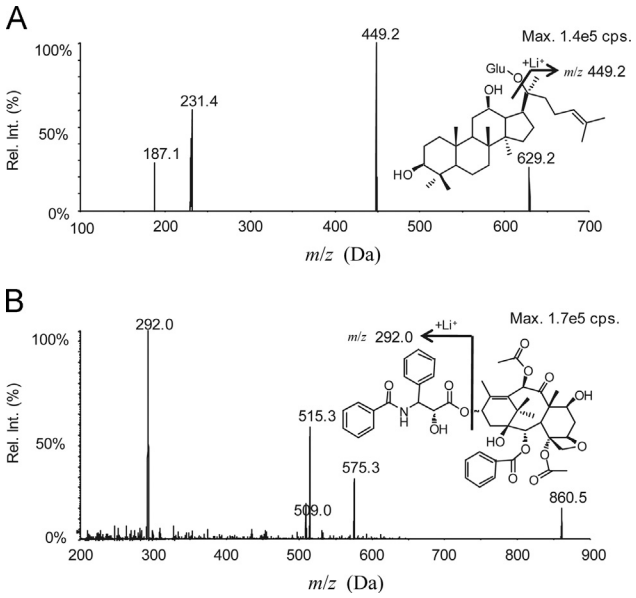


Figure 2 Product ion spectra of $[M+Li]^+$ of (A) GCK and (B) paclitaxel as well as proposed fragmentation patterns.

GCK only 0.2 mmol/L lithium carbonate was needed to generate a stable $[M+Li]^+$ signal, presumably because the water content of the mobile phase was relatively low. A column temperature of 40 °C and a flow rate of 0.5 mL/min also contributed to producing symmetrical peaks for GCK and paclitaxel with retention time of only 1.5 and 1.3 min, respectively.

3.3. *Sample preparation*

Sample preparation by protein precipitation was initially evaluated, but strong matrix effects ruled it out. Liquid–liquid extraction (LLE) with various solvents (methyl *tert*-butyl ether, ethyl acetate, *n*-hexane, *n*-hexane/dichloromethane/isopropanol 2:1:0.1, *v/v/v*) was then investigated and methyl *tert*-butyl ether gave the highest recovery.

3.4. *Method validation*

3.4.1. *Selectivity*

Fig. 3 presents typical chromatograms of blank plasma, plasma spiked with IS, plasma spiked with GCK at the LLOQ and IS, and a plasma sample collected 1.5 h after oral administration of a GCK

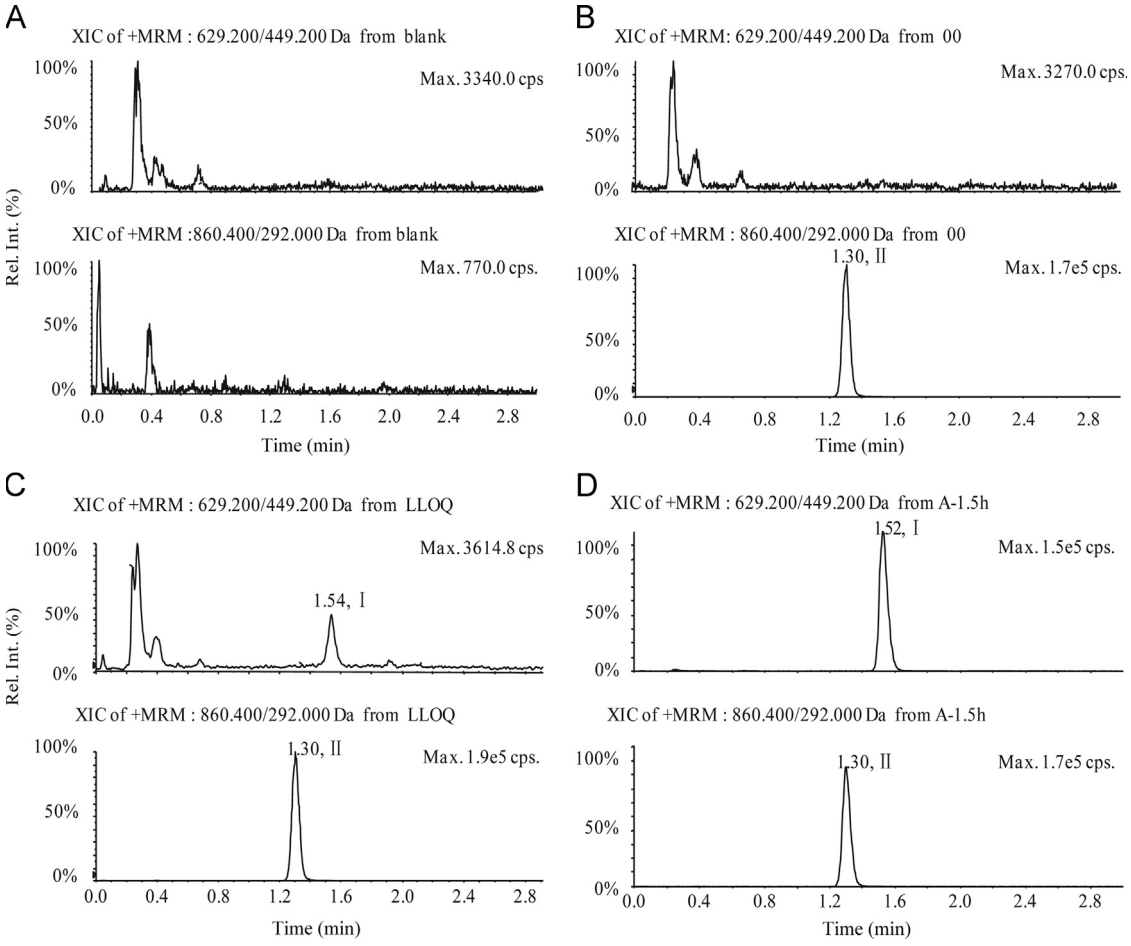


Figure 3 Representative MRM chromatograms of (I) GCK and (II) paclitaxel in (A) blank plasma, (B) plasma spiked with paclitaxel (200 ng/mL), (C) plasma sample spiked with GCK (1.0 ng/mL) and paclitaxel (200 ng/mL) and (D) a plasma sample collected 1.5 h after oral administration of a GCK 50 mg tablet.

Table 1 Precision and accuracy data for analysis of GCK in human plasma.

Nominal conc. (ng/mL)	Mean ± SD (ng/mL)	Inter-day RSD (%)	Intra-day RSD (%)	Accuracy as RE (%)
1.00	0.99 ± 0.05	8.4	4.7	−1.35
2.00	1.93 ± 0.08	3.6	4.2	−3.36
30.0	29.0 ± 1.0	4.3	3.2	−2.78
800	835 ± 27	6.0	2.6	4.32

Data are based on the assay of six replicates per day on three separate days.

Table 2 Recovery and matrix effects for GCK and paclitaxel (IS).

Compd.	Nominal conc. (ng/mL)	Recovery		Matrix effects	
		Mean ± SD (%)	RSD (%)	Mean ± SD (%)	RSD (%)
GCK	2.00	80.0 ± 2.4	3.8	109 ± 4	3.5
	30.0	81.9 ± 3.9	3.8		
	800	83.4 ± 0.9	1.1	111 ± 3	2.7
IS	200	81.9 ± 3.9	1.6		

Data are based on the assay of six samples of blank non-hemolyzed plasma and one sample of hemolyzed plasma.

Table 3 Stability of GCK in human plasma and processed samples under various storage conditions ($n=3$).

Storage condition	Concentration (ng/mL)		RSD (%)
	Nominal	Mean ± SD	
−80 °C/32 days	2.00	1.88 ± 0.09	−6.0
	800	778 ± 13	−2.8
−80 °C/3 freeze-thaw cycles	2.00	1.85 ± 0.04	−7.4
	800	771 ± 11	−3.6
Room temperature/6 h	2.00	1.93 ± 0.20	−3.3
	800	781 ± 10	−2.4
4 °C/24 h (processed samples)	2.00	2.10 ± 0.13	3.2
	800	789 ± 9	−1.3

50 mg tablet. The *S/N* ratio at the LLOQ was >10.3 and analyte and IS were free of interference from endogenous substances in both non-hemolyzed and hemolyzed plasma.

3.4.2. *Linearity and LLOQ*

The assay was shown to be linear in the range 1.00–1000 ng/mL with a typical linear regression equation of $y=0.00631x+0.00112$ ($r=0.9989$). The LLOQ of GCK was 1.00 ng/mL.

3.4.3. *Precision and accuracy*

Intra- and inter-day precision and accuracy data at QC concentrations are summarized in Table 1. The results demonstrate that accuracy and precision are within acceptable ranges.

3.4.4. *Recovery and matrix effects*

Recovery of GCK from low, medium and high QC samples and of the IS are shown in Table 2. In all cases recovery was ≥80.0%.

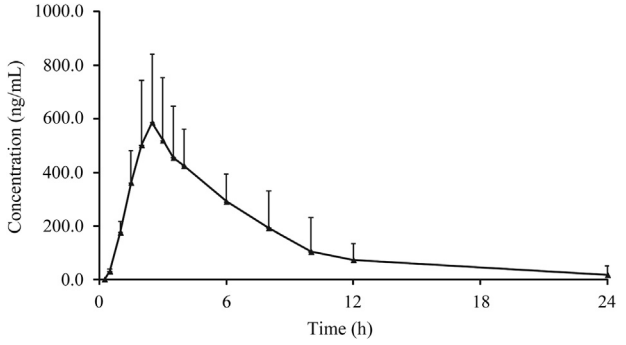


Figure 4 Mean plasma concentration-time profile of GCK after single oral administration of a 50 mg tablet to healthy volunteers (data are mean ± SD, $n=12$).

Evaluation of matrix effects indicate concentrations were 109% and 111% of nominal low and high concentrations and were not significant issue.

3.4.5. *Stability*

The results of the stability tests of GCK in plasma and processed samples are shown in Table 3. GCK was shown to be stable under all storage conditions tested. The stock solution of GCK and working solution of IS were also found to be stable under the conditions tested.

3.4.6. *Carry-over*

No significant carry-over was observed for the analyte and IS when analyzing blank plasma immediately after the highest calibration standard (1000 ng/mL).

3.5. *Pharmacokinetic study*

The assay was successfully applied to a pharmacokinetic study in 12 healthy Chinese volunteers after oral administration of a GCK

50 mg tablet. The mean plasma concentration–time profile of GCK is shown in Fig. 4. Non-compartmental pharmacokinetic parameters calculated using WinNonlin 6.1 were as follows: C_{max}, 652 ± 180 ng/mL; T_{max}, 2.63 ± 1.17 h; $t_{1/2}$, 5.97 ± 0.68 h; $AUC_{(0-t)}$, 3650 ± 850 ng · h/mL; $AUC_{(0-\infty)}$, 3810 ± 890 ng · h/mL.

3.6. Comparison with previous methods

The only previously reported LC–MS/MS assay for GCK in human plasma used the negative ion mode and digoxin as IS[13]. The present method has the following advantages: (1) The sensitivity is substantially better due to the MRM of $[M+Li]^+$ ions instead of $[M-H]^-$; (2) the LLOQ of 1 ng/mL is achieved using 50 μL plasma instead of 100 μL; and (3) the method is more accurate, precise and reproducible than the previous method, probably because the difference in polarity between GCK and paclitaxel is smaller than between GCK and digoxin and because the IS was added before but not after LLE.

4. Conclusions

An LC–MS/MS method for determination of GCK in human plasma based on formation of lithium adducts of the analyte and IS (paclitaxel) has been developed and validated. The assay is rapid and sensitive and has the potential to allow relatively high sample throughput and to be useful in the assay of other neutral compounds. The method was successfully applied to a pharmacokinetic study involving a single oral administration of GCK to healthy Chinese volunteers.

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